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IMMUNISATION OF ANIMALS AGAINST INFECTION BY *VIBRION SEPTIQUE* AND *BACILLUS CHAUVŒI.*

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It has long been recognised that the domesticated animals, especially cattle and sheep, are subject to acute, and usually fatal, infection by both *Vibrion septique* and *Bacillus chauvœi*. Cases of disease in horses and swine have also been ascribed, though with less frequency, to one or other of these organisms. Before passing on to deal with these particular infections in animals a few observations on anaerobes in general are perhaps necessary.

Prior to the war there appears to have existed a great deal of confusion regarding the real differences between the various groups of anaerobes. A few workers undoubtedly were aware of these differences, but they were vastly outnumbered by those who were not. Pure cultures were thus extremely rare, and most of the descriptions then given are consequently of little value at the present time. As a result, however, of the large amount of work carried out during the war period and of the improved technique evolved, the subject is now in a much more satisfactory state. Particularly is this the case in so far as *V. septique* is concerned. The use of the term "malignant œdema bacillus" applied to a proteolytic cutlure is now less frequently seen in the literature than formerly. But as an indication that the differences between groups of anaerobes are not universally appreciated even now, we may mention that during the

* This work was carried out at the Wellcome Physiological Research Laboratories.

course of this work we have received from various laboratories four cultures of supposed *B. chauvœi* all of which contained *V. septique*, in three cases in pure culture, in one contaminated with *B. sporogenes*. For a clear description of the main characters of *V. septique* and *B. chauvœi* we would refer to the article published in 1920 by Heller (1), which contains an excellent critical review of the literature dealing with the anaerobic infections of animals together with the results of her own examinations of many strains of anaerobes obtained from animal material.

We have studied fifteen strains that were isolated from cases diagnosed clinically as blackleg, and found that ten were *B. chauvœi* and five *V. septique*. Of the *B. chauvœi* strains, five were obtained from various laboratories and five were isolated by us from fresh muscle. Of the strains which proved to be *V. septique*, four were sent to us as *B. chauvœi* and one was isolated from fresh muscle. The six specimens of fresh muscle were sent to us by veterinary surgeons from cases which they had diagnosed as clinical blackleg. *B. chauvœi* was isolated from five of these. In the remaining case we searched very thoroughly for this organism, but without success, though we tried all the methods at our disposal to obtain it in culture. On the other hand, we had no difficulty in isolating *V. septique*. The muscle had all the appearances of blackleg muscle and possessed the sour but not putrefactive odour which we have been taught to regard as typical of such cases. In the specimens from which we isolated *B. chauvœi* no *V. septique* was present.

Our strains agree very closely in all their essential characters with the descriptions given by Heller (*loc. cit.*), and as these are now so well known it is quite unnecessary for us to enter into all the details. We should like to repeat, however, that both organisms are non-proteolytic, neither digesting nor blackening meat or brain media, and they do not exhale a putrid odour. In pure culture they may readily be distinguished the one from the other by a consideration of their colony formation, sugar fermentations, lesions produced in experimental animals, and by the property possessed by *V. septique*, in contrast to *B. chauvœi*, of forming filaments on the surface of the liver in such animals.

We were particularly interested in the fact that a proportion of our strains proved to be *V. septique*, as until comparatively recently it was generally believed that blackleg was a specific disease attributable solely to infection by *B. chauvœi*. It may be too early yet to assert that such is not the case, but it must be admitted that evidence to the contrary increases year by year.

Heller examined thirty-two strains of anaerobes, all of which were

isolated from clinical cases of blackleg in cattle. She found that nineteen of these were *B. chauvœi* and thirteen *V. septique*. Further, she made a thorough review of the literature dealing with the subject, and, as a result, states: "Practical considerations lead to the following conclusions: cattle are frequently subject to spontaneous infection by members of two groups of anaerobic invaders, the blackleg group and the *V. septique* group. Both types of these infections are, in the vast majority of cases, diagnosed as blackleg, Rauschbrand or symptomatic anthrax. Infection by members of the first group is commoner than by members of the second group, but infection by members of the second group is by no means to be ignored, and probably should be considered in cattle immunisation. It is possible that a member of one group may be the predominating invader in one district and a member of the other in another, which indicates the necessity of determining the bacterial agents involved in blackleg infection in various districts."

Uchimura (2) divides his fifteen Rauschbrand strains into eleven true Rauschbrand, and four others, of which two were *V. septique* and two showed proteolytic characters. St. Ivanic (3) classifies his Rauschbrand strains into an atoxic and a toxic group, i.e. probably *B. chauvœi* and *V. septique*, for he states that the toxin of his toxic group is neutralised by what he calls antitoxic œdema serum prepared by the National Sero-therapeutic Institute in Vienna.

Graub (4), in discussing the results of immunising a number of cattle in Switzerland with germ-free blackleg filtrate, points out that *V. septique* was responsible for fifteen out of forty-eight cases in which the natural disease developed in animals so immunised. He moreever quotes the authority of Gabathuler of Davos and of Gerlach of Vienna for the statement that this organism is the causal agent of some cases of blackleg in the Canton of Graubund and in the Tyrol respectively. In Germany two types of Rauschbrand are apparently recognised: Fothscher Rauschbrand or true Rauschbrand and Kittscher Rauschbrand, which is caused by what is there known as the malignant œdema bacillus (probably *V. septique*). The State pay compensation to the owner for the loss of animals in the case of true Rauschbrand only.

Leclainche and Vallée (5) (*a*) distinguish between blackleg and gas gangrene in animals, and state that cases of the latter condition are due to wound infection by *V. septique*, as, for example, following parturition or in cases where there are abrasions of the tongue, pharynx, or teats. Among cases of true blackleg arising in the absence of any discoverable wound, they have met with some in which the causal organisms have shown themselves to be neither true

B. chauvœi nor true *V. septique*, but to be possessed of certain characters of both. They do not, however, recognise true *V. septique* as a cause of blackleg.

Sheep are known to be the subjects of blackleg, particularly in certain districts. Many cases have been shown to be due to infection with *B. chauvœi*, but here again it is possible that *V. septique* is sometimes responsible for the condition. In sheep, however, the latter organism assumes greater importance than it does in cattle on account of its significance in the disease known as braxy or bradsot. The first worker to state definitely that *V. septique* was the specific cause of bradsot was Zeissler (6), who described the organism under the name of the bacillus of Ghon and Sachs.

Notable earlier investigations into the nature of this disease were made by Neilsen (7) and by Jensen (8). The latter described his cultures very carefully, but did not identify them as *V. septique*. From eleven samples of bradsot material sent to her by Jensen, Heller isolated *V. septique* from seven, *B. chauvœi* from one, and in the remaining three she found no pathogenic organisms. Later Gaiger (9), in an account of his investigations in Scotland, confirms the view that *V. septique* is the cause of braxy, although his description of the nine strains which he studied in detail is not completely satisfactory as far as sugar fermentations are concerned. He states "that it was found to be very difficult to obtain any uniformity in the results which varied in different sub-cultures even of the same strain." Only four of these nine strains fermented salicin and lactose. Gaiger commits himself to the statement that "he had formed the general opinion that fermentation tests are uncertain and unreliable for the differentiation of pathogenic anaerobes." We can only say that we have found such tests very reliable for all our strains, both of *V. septique* and of *B. chauvœi*, and we believe that given pure cultures they are just as valuable in the differentiation of anaerobes as of aerobes, no more and no less. All the strains of *V. septique*, both of human and animal origin, which we have examined have fermented salicin and lactose with avidity.

Immunity.

The earlier measures adopted for the preventive inoculation of animals against *V. septique* and *B. chauvœi* have all involved the use of living organisms—either fully virulent or in an attenuated form—with or without the use of anti-serum. Examples which may be given are the classical methods of Arloing, of Kitt and of Leclainche and Vallée in the case of blackleg, and of Jensen in the

case of braxy. All of these have given good results, and are more or less widely used at the present time. Of late years, however, for obvious reasons, attention has been focussed on the value of germ-free filtrates as immunising agents against these organisms. It was Roux (10) who first pointed out in 1888 that filtrates of cultures of *V. septique* and *B. chauvœi* were efficient for immunising guinea-pigs, and that filtered serous fluid of animals dead of these infections was equally serviceable for this purpose. His methods were not, however, adopted on a large scale until comparatively recently, when other workers, notably Schöbl (11), Nitta (12), Eichhorn (13), and Graub and Zschokke (14) have testified to their efficacy. In so far as *B. chauvœi* is concerned it is now accepted that such filtrates can be relied upon to induce a high degree of immunity against natural infection.

The only reference which we can find, apart from Roux's original work, to the use of a filtrate as an immunising agent against *V. septique* infection is contained in the article by Gaiger to which we have already referred. Gaiger found that an injection of one or two doses of *V. septique* toxin in sheep enabled them to withstand a lethal dose of culture two or three weeks later, but that such animals were not immune when tested in six weeks' time—a result which is not in accordance with ordinary immunological experience. The same worker also used the toxin as a prophylactic against natural braxy infection, and records a considerable diminution in losses from this disease among sheep thus inoculated.

Our own efforts have been directed entirely to the immunisation of animals against *V. septique* and *B. chauvœi* by means of filtrates of artificial culture.

The medium which we have found most satisfactory for the production of good filtrates is a tryptic digest broth to which fresh minced muscle is added. The technique adopted in the preparation of the broth is very similar to that employed by Hartley (15), and is as follows :—

Fourteen kilogrammes of meat, from which fat and connective tissue have been removed as far as possible, are distributed into four buckets, 3·5 kilos per bucket, and 3 litres of tap water are added for every 2 kilos of meat. The meat is well mixed with the water and the buckets allowed to stand at room temperature overnight (16 to 18 hours). Next morning the contents of the buckets are steamed to 80° C. to destroy any antitryptic action of the muscle. The contents of each are then placed in large earthenware vessels and cooled to 45° C. by the addition of 0·8 per cent. anhydrous sodium carbonate solution. To bring the brew to a suitable volume and a

PH of about 9·2, 3 litres of this solution are required per 2 kilogrammes of muscle. The trypsin preparation and the preservative are now added. Forty cubic centimetres of the trypsin extract per kilogramme of meat used has been found to give satisfactory results. As a preservative during digestion, chloroform (30 c.c. per 2 kilos of meat) is the most satisfactory owing to the ease with which it is eventually removed.

The digestion is allowed to proceed for six hours in the incubator at 37° C. with frequent stirring. The action of the trypsin is then stopped by the addition of normal hydrochloric acid to the brew bulked in a large cauldron provided with a steam jacket (350 c.c. acid per 14 kilos muscle). The destruction of the trypsin is completed by heating the brew to 100° C. and then filtering. It is important to filter as soon as possible after the digestion is completed and the enzyme destroyed. Filtration at this stage is very rapid, and the brew may be left in the acid condition overnight.

Early next morning the reaction is made alkaline by the addition of normal sodium hydroxide. The broth is distributed in quantities of 1,800 c.c. into Winchester quarts, to each of which 150 grams of fresh minced muscle (horseflesh or beef) are added and thoroughly mixed with the broth. The temperature is then raised to 100° C. When cool the broth is poured off and its reaction adjusted to PH 8. It is again heated to 100° C. to precipitate phosphates, filtered, poured back on to the meat and autoclaved, free steam being allowed to pass through for sixty minutes, after which the pressure is raised slowly to 10 pounds and maintained there for 20 minutes. The final reaction of the medium is approximately PH 7·8. The trypsin preparation has been obtained from fresh pancreas of the pig according to the directions of Cole and Onslow (16).

It has been our custom to inoculate each bottle of medium with 30 c.c. of a 24–48 hours' broth culture of the organism, and to add at the same time sufficient sterile glucose solution to give a concentration of 0·2 per cent. Growth takes place very actively in this medium at body temperature and is allowed to proceed for two to four days in the case of *V. septique* and for five days in the case of *B. chauvœi*, when filtration through Berkefeld candles is practised.

"Vibrion Septique" Filtrate or Toxin.

Filtered culture of *V. septique* contains a toxin which can easily be demonstrated by animal inoculation. When the organism is grown in the medium which we have described the toxin reaches its maximum strength in from two to four days, after which, if kept in

the incubator, it gradually declines until after an incubation period of twenty days the filtrate is almost atoxic. We have found that the toxin remains very constant in potency so long as it is kept in a cold room and in the dark. One of our toxins so preserved is of practically the same value to-day as when it was first filtered eleven months ago. At room temperature and exposed to the light it deteriorates much more quickly.

The general properties of the toxin have been fully described by Robertson (17). It produces rapid death in animals by intravenous inoculation, the symptoms shown being those of respiratory disturbance, paralysis and convulsions. It is not fatal by subcutaneous inoculation unless large quantities are given, but it does cause marked local reaction in the form of swelling and exudate followed by extensive necrosis. The intravenous inoculation of rabbits may be practised in estimating the potency of a given toxin, but where large numbers of tests have to be carried out this method becomes very expensive, and it is better to use smaller animals.

Most of our titrations have been carried out quite satisfactorily in mice. Guinea-pigs may be used for the purpose and are equally good, but in them the actual injection is more troublesome as it necessitates anæsthetising the animal and making an incision through the skin. We have found that the most convenient vein for this purpose lies on the outer side of the leg just above the hock, whence it runs in an upward and inward direction over the back of the limb.

TABLE 1.—TITRATION OF "VIBRION SEPTIQUE" TOXIN INTRAVENOUSLY IN MICE.

Mouse.	Weight.	Dose.	Result.
A	17 grams	0·25 c.c.	Died 3 mins.
B	17 grams	0·25 c.c.	Died 2 mins.
C	16 grams	0·1 c.c.	Died 5 mins.
D	18 grams	0·1 c.c.	Died 4 mins.
E	17 grams	0·05 c.c.	Died 55 mins.
F	16 grams	0·05 c.c.	Died 50 mins.
G	17 grams	0·025 c.c.	Died 130 mins.
H	17 grams	0·025 c.c.	Died 114 mins.
J	18 grams	0·01 c.c.	Died 48 hrs. (approx.).
K	17 grams	0·01 c.c.	Died 24 hrs. (approx.).
L	16 grams	0·005 c.c.	Died 72 hrs. (approx.).
M	17 grams	0·005 c.c.	Died 72 hrs. (approx.).
N	18 grams	0·0025 c.c.	Survived.
O	17 grams	0·0025 c.c.	Survived.
P	16 grams	0·001 c.c.	Survived.
Q	17 grams	0·001 c.c.	Survived.

A typical titration of *V. septique* toxin intravenously in mice is shown in Table 1 and in guinea-pigs in Table 2. Of this toxin 0·1 c.c. was sufficient to kill a medium sized rabbit, so that a comparison shows that the minimum lethal dose of toxin for a guinea-pig is about ten times, and for a medium-sized rabbit about twenty times that for a mouse. A glance at Table 1 will show that where mice of approximately equal weights are used, the time elapsing between injection and death increases as the dose decreases.

TABLE 2.—TITRATION OF "VIBRION SEPTIQUE" TOXIN INTRAVENOUSLY IN GUINEA-PIGS.

Guinea-Pig.	Weight.	Dose.	Time.	Result.
A	420 grams	0·05 c.c.	11.50 a.m.	Died overnight.
B	375 grams	0·06 c.c.	11.55 a.m.	Found dead 4.55 p.m.
C	390 grams	0·07 c.c.	12.0 noon.	Died 2.31 p.m.
D	415 grams	0·08 c.c.	12.5 p.m.	Died 2.30 p.m.
E	390 grams	0·09 c.c.	12.8 p.m.	Died 1.30 p.m.
F	380 grams	0·1 c.c.	12.12 p.m.	Died 12.33 p.m.
G	400 grams	0·15 c.c.	12.15 p.m.	Died 1.18 p.m.
H	400 grams	0·2 c.c.	12.19 p.m.	Died 12.26 p.m.
J	400 grams	0·25 c.c.	12.24 p.m.	Died 12.30 p.m.

TABLE 3.—TITRATION OF "VIBRION SEPTIQUE" TOXIN AGAINST ANTITOXIN INTRAVENOUSLY IN MICE.

Mouse.	Dose.			Result.
	"V. Septique" Toxin		*Antitoxin.*	
A	0·5 c.c.	+	0·003125 c.c.	Died 3 mins.
B	0·5 c.c.	+	0·00625 c.c.	Died 6 mins.
C	0·5 c.c.	+	0·0125 c.c.	Delayed death.
D	0·5 c.c.	+	0·025 c.c.	Lived.
E	0·5 c.c.	+	0·05 c.c.	Lived.
F	0·5 c.c.	+	0·1 c.c.	Lived.
Controls.				
G	0·5 c.c.		Toxin only	Died 3 mins.
H	0·5 c.c.		Toxin only	Died 5 mins.
J	0·25 c.c.		Toxin only	Died 5 mins.
K	0·25 c.c.		Toxin only	Died 7 mins.
L	0·5 c.c.		Toxin + 0·2 c.c. normal serum	Died 3 mins.

We have used the same methods for titrating a toxin against a known antitoxin in an endeavour to find the point of neutrality

before preparing toxin antitoxin mixtures for immunising purposes. Tables 3 and 4 show such titrations in mice and guinea-pigs respectively.

Table 4.—Titration of "Vibrion Septique" Toxin against Antitoxin Intravenously in Guinea-Pigs.

Guinea-Pig.	Dose.			Result.
	"V. Septique" Toxin.		*Antitoxin.*	
A	1 c.c.	+	0·00625 c.c.	Died 7 mins.
B	1 c.c.	+	0·0125 c.c.	Died overnight.
C	1 c.c.	+	0·025 c.c.	Lived.
D	1 c.c.	+	0·05 c.c.	Lived.
Controls.				
E	0·75 c.c.		Nil	Died 4 mins.
F	1 c.c.	+	0·2 c.c. normal serum	Died 3 mins.

Table 5 represents the titration of the same toxin and antitoxin with a view to testing the local effect of the subcutaneous injection of mixtures of varying strength. A comparison with Table 4 shows that the point of neutrality of the mixtures is indicated equally well for most practical purposes by either of these methods, although for really accurate work we should prefer the intravenous injection.

Table 5.—Titration of "Vibrion Septique" Toxin Antitoxin Mixtures for Local Effect Subcutaneously in Guinea-Pigs.

Guinea-Pig.	Dose.			Result.
	"V. Septique" Antitoxin.		*Toxin.*	
A	0·02 c.c.	+	2 c.c.	Swelling and exudate.
B	0·02 c.c.	+	2 c.c.	Swelling and exudate.
C	0·03 c.c.	+	2 c.c.	Slight swelling and exudate.
D	0·03 c.c.	+	2 c.c.	Slight swelling and exudate.
E	0·04 c.c.	+	2 c.c.	Slight swelling only.
F	0·04 c.c.	+	2 c.c.	Slight swelling only.
G	0·05 c.c.	+	2 c.c.	Nil.
H	0·05 c.c.	+	2 c.c.	Nil.

Immunisation of Guinea-Pigs against "V. Septique."

Our first attempts in this direction consisted in the subcutaneous injection of toxin, but although the animals acquired a reasonable

degree of immunity after two doses had been given, we were compelled to abandon this method as a practical measure owing to the serious local effects caused by the administration. We were obliged therefore to obviate these effects either by using a mixture of toxin and antitoxin, or by so modifying the toxin as to reduce its toxicity to a minimum whilst at the same time preserving its immunising properties. Both of these methods proved successful.

TABLE 6.—IMMUNISATION OF GUINEA-PIGS WITH SINGLE DOSE OF "VIBRION SEPTIQUE" MIXTURE (50 PER CENT. UNDER-NEUTRALISED).

Guinea-Pig.	Inoculated, 23/10/23.	Tested, 13/11/23.	Result.
	"V. Septique" Mixture.	*"V. Septique" Toxin I.V.*	
A	(0·02 c.c. antitoxin + 2 c.c. toxin)	2 M.L.D. (0·1 c.c.)	Lived.
B	(0·02 c.c. antitoxin + 2 c.c. toxin)	2 M.L.D. (0·1 c.c.)	Lived.
C	(0·02 c.c. antitoxin + 2 c.c. toxin)	2 M.L.D. (0·1 c.c.)	Died 48 hours.
D	(0·02 c.c. antitoxin + 2 c.c. toxin)	5 M.L.D. (0·25 c.c.)	Died 16 mins.
E	(0·02 c.c. antitoxin + 2 c.c. toxin)	5 M.L.D. (0·25 c.c.)	Died 1 hour (approx.).
Controls.			
F	Nil	2 M.L.D. (0·1 c.c.)	Died overnight.
G	Nil	2 M.L.D. (0·1 c.c.)	Died 5 hours.
H	Nil	5 M.L.D. (0·25 c.c.)	Died 13 mins.

As was to be expected, we found that a double dose gave a much stronger immunity than a single one. A comparison of Tables 6 and 7 illustrates this point in the case of a toxin antitoxin mixture. From these it will be seen that we employed two methods as a test of immunity: (*a*) The intramuscular injection of culture and (*b*) the intravenous injection of toxin. We have found the intravenous injection of toxin to be just as reliable as a dose of culture in testing immunity, and we prefer its use, since the strength of the amount given can be much more accurately measured than is the case with culture.

When two doses are given the first may consist of a toxin anti-

TABLE 7.—IMMUNISATION OF GUINEA-PIGS WITH DOUBLE INOCULATION OF 50 PER CENT. UNDER-NEUTRALISED "VIBRION SEPTIQUE" TOXIN ANTITOXIN MIXTURE.

Guinea-Pig.	1st Inoculation, 23/10/23.	2nd Inoculation, 5/11/23.	Tested, 20/11/23.	Result.
	"V. Septique" Mixture.	*"V. Septique" Mixture.*	*"V. Septique" Toxin IV.*	
A	2 c.c.	2 c.c.	5 M.L.D. (0·25 c.c.)	Lived.
B	2 c.c.	2 c.c.	5 M.L.D. (0·25 c.c.)	Lived.
C	2 c.c.	2 c.c.	5 M.L.D. (0·25 c.c.)	Lived.
D	2 c.c.	2 c.c.	8 M.L.D. (0·4 c.c.)	Lived.
E	2 c.c.	2 c.c.	8 M.L.D. (0·4 c.c.)	Died 7 mins.
Controls.				
F	Nil	Nil	2 M.L.D. (0·1 c.c.)	Died overnight.
G	Nil	Nil	2 M.L.D. (0·1 c.c.)	Died overnight.
H	Nil	Nil	5 M.L.D. (0·25 c.c.)	Died 10 mins.
J	Nil	Nil	10 M.L.D. (0·5 c.c.)	Died 7 mins.
			28/11/23	
	"V. Septique" Mixture.	*"V. Septique" Mixture.*	*"V. Septique" Culture I.M.*	
K	2 c.c.	2 c.c.	0·05 c.c.	Lived.
L	2 c.c.	2 c.c.	0·05 c.c.	Lived.
Control.				
M	Nil	Nil	0·05 c.c.	Died 24–48 hours.
			1/12/23	
	"V. Septique" Mixture.	*"V. Septique" Mixture.*	*"V. Septique" Culture I.M.*	
N	2 c.c.	2 c.c.	0·05 c.c.	Lived.
O	2 c.c.	2 c.c.	0·05 c.c.	Lived.
P	2 c.c.	2 c.c.	0·05 c.c.	Lived.
Controls.				
Q	Nil	Nil	0·05 c.c.	Died 24–48 hours.
R	Nil	Nil	0·05 c.c.	Died 24 hours.

TABLE 8.—IMMUNISATION OF GUINEA-PIGS WITH "VIBRION SEPTIQUE" TOXIN ANTITOXIN MIXTURE AND TOXIN ALONE.

Guinea-Pig.	1st Inoculation, 15/9/23.	2nd Inoculation, 1/10/23.	Test, 9/10/23.	Result.
	2 c.c. Neutral T.A.M." V. Septique" Antitoxin Toxin.	*"V. Septique" Toxin.*	*"V. Septique" Toxin I.V.*	
A	0·5 c.c. + 2 c.c.	0·5 c.c.	2 M.L.D. (0·1 c.c.)	Lived.
B	0·5 c.c. + 2 c.c.	0·5 c.c.	5 M.L.D. (0·25 c.c.)	Died 48 hours.
C	0·5 c.c. + 2 c.c.	0·3 c.c.	2 M.L.D. (0·1 c.c.)	Lived.
D	0·5 c.c. + 2 c.c.	0·3 c.c.	5 M.L.D. (0·25 c.c.)	Lived.
E	0·5 c.c. + 2 c.c.	0·2 c.c.	2 M.L.D. (0·1 c.c.)	Lived.
F	0·5 c.c. + 2 c.c.	0·2 c.c.	5 M.L.D. (0·25 c.c.)	Lived.
Control. G	Nil	Nil	5 M.L.D. (0·25 c.c.)	Died 70 mins.

toxin mixture and the second of a small quantity of neat toxin, for in response to the first dose the animal acquires sufficient immunity to protect it from the serious local effects of the toxin. Table 8 shows the result of such an experiment.

In the preparation of mixtures for immunising purposes the proportion of serum added should be the least amount necessary to prevent too great a local reaction. A comparison of Tables 9 and 10 shows that, of two mixtures in which the same amount of toxin was used, the one containing the lesser amount of serum gave the higher degree of immunity.

MODIFIED TOXIN.

By adding a small percentage of formalin to a batch of toxin and keeping it at a temperature of 30° C. for a week we obtained an atoxic product which retained its immunising properties as shown in Table 11. We think we ought to state, however, that until we have completed some further experiments, we do not feel that we are in a position to define the exact conditions necessary to bring about this result. The danger is that the toxic and immunising properties may disappear simultaneously.

"BACILLUS CHAUVŒI" FILTRATE.

The filtrates which we have prepared from cultures of this organism have all proved to be quite atoxic for animals by whatever

TABLE 9.—IMMUNISATION OF GUINEA-PIGS WITH A 25 PER CENT. UNDER-NEUTRALISED "VIBRION SEPTIQUE" MIXTURE. DOUBLE INOCULATION.

Guinea-Pig.	1st Inoculation, 5/11/23.	2nd Inoculation, 28/12/23.	Tested, 4/3/24.	Result.
	"V. Septique" Mixture.	*"V. Septique" Mixture.*	*Toxin I.V.*	
A	2 c.c.	2 c.c.	2 M.L.D. (0·1 c.c.)	Lived.
B	2 c.c.	2 c.c.	2 M.L.D. (0·1 c.c.)	Lived.
C	2 c.c.	2 c.c.	5 M.L.D. (0·25 c.c.)	Lived.
D	2 c.c.	2 c.c.	5 M.L.D. (0·25 c.c.)	Died 24 hours.
E	2 c.c.	2 c.c.	5 M.L.D. (0·25 c.c.)	Died 15 mins.
Controls.				
F	Nil	Nil	2 M.L.D. (0·1 c.c.)	Died 14 mins.
G	Nil	Nil	2 M.L.D. (0·1 c.c.)	Died 24 hours.
H	Nil	Nil	2 M.L.D. (0·1 c.c.)	Died 24 hours.
J	Nil	Nil	2 M.L.D. (0·1 c.c.)	Died 4 hours.
K	Nil	Nil	5 M.L.D. (0·25 c.c.)	Died 8 mins.
L	Nil	Nil	10 M.L.D. (0·5 c.c.)	Died 2 mins.

channel they have been introduced. This is in accordance with the findings of most workers on the subject. On the other hand, *B. chauvœi* toxin has been prepared, notably by Leclainche and Vallée (5) (*b*), who first described its properties in 1900. Quite recently Okuda (18) has been successful in obtaining a similar toxin. Apparently, however, the majority of strains of this organism are non-toxigenic. Haslam and Lumb (19) consider that the immunising power of such filtrates is not dependent upon toxicity. However that may be, it is now established that atoxic filtrates of this organism may be quite efficient as immunising agents.

Unfortunately, in the absence of a demonstrable toxin, there does not exist at the moment any reliable quick method of estimating the value of a given filtrate. An endeavour has been made by Scott (20) to devise means whereby this can be done. He compares the "aggressive" power of various filtrates by testing the amounts

TABLE 10.—IMMUNISATION OF GUINEA-PIGS WITH A 50 PER CENT. UNDER-NEUTRALISED "VIBRION SEPTIQUE" MIXTURE. DOUBLE INOCULATION.

Guinea-Pig	1st Inoculation 5/11/23.	2nd Inoculation, 28/11/23.	Tested, 4/3/24.	Result.
	"V. Septique" Mixture.	*"V. Septique" Mixture.*	*Toxin I.V.*	
A	2 c.c.	2 c.c.	2 M.L.D. (0·1 c.c.)	Lived.
B	2 c.c.	2 c.c.	2 M.L.D. (0·1 c.c.)	Lived.
C	2 c.c.	2 c.c.	5 M.L.D. (0·25 c.c.)	Lived.
D	2 c.c.	2 c.c.	10 M.L.D. (0·5 c.c.)	Lived.
Controls.				
E	Nil	Nil	2 M.L.D. (0·1 c.c.)	Died 14 mins.
F	Nil	Nil	2 M.L.D. (0·1 c.c.)	Died 24 hours.
G	Nil	Nil	2 M.L.D. (0·1 c.c.)	Died 24 hours.
H	Nil	Nil	2 M.L.D. (0·1 c.c.)	Died 4 hours.
J	Nil	Nil	5 M.L.D. (0·25 c.c.)	Died 8 mins.
K	Nil	Nil	10 M.L.D. (0·5 c.c.)	Died 2 mins.

of them that must be added to a given quantity of an emulsion of washed *B. chauvœi* spores to produce death when inoculated into guinea-pigs. In our hands this did not prove very satisfactory owing chiefly to the varying susceptibility that exists among individual guinea-pigs towards infection by this organism. It may be said, however, to provide some slight indication. The accurate standardisation of *B. chauvœi* filtrate is therefore impossible at the present time. Each batch of the material must be tested for immunising power on animals.

IMMUNISATION OF GUINEA-PIGS WITH "BACILLUS CHAUVŒI" FILTRATE.

Tables 12 and 13 show typical examples of the results which we have obtained in immunising guinea-pigs with *B. chauvœi* filtrate. Here again it will be seen that the inoculation of two doses stimulates the production of a higher degree of immunity than does a single dose.

TABLE 11.—IMMUNISATION OF GUINEA-PIGS WITH TWO DOSES OF "VIBRION SEPTIQUE" TOXIN MODIFIED BY ADDING 0·3 PER CENT. FORMALIN AND MAINTAINING AT A TEMPERATURE OF 30° C. FOR A WEEK.

Guinea-Pig.	1st Inoculation, 13/10/23.	2nd Inoculation, 25/11/23.	Tested 14 Days Later.	Result.
	"V. Septique" Toxin.	*"V. Septique" Toxin.*	*"V. Septiqve" Toxin IV.*	
A	2 c.c. toxin subcutaneously	2 c.c. toxin subcutaneously	2 M.L.D. (0·1 c.c.)	Lived.
B	2 c.c. toxin subcutaneously	2 c.c. toxin subcutaneously	5 M.L.D. (0·25 c.c.)	Lived.
C	2 c.c. toxin subcutaneously	2 c.c. toxin subcutaneously	5 M.L.D. (0·25 c.c.)	Lived.
D	2 c.c. toxin subcutaneously	2 c.c. toxin subcutaneously	5 M.L.D. (0·25 c.c.)	Died 48 hours.
Controls.				
E	Nil	Nil	2 M.L.D. (0·1 c.c.)	Died overnight.
F	Nil	Nil	2 M.L.D. (0·1 c.c.)	Died overnight.
G	Nil	Nil	2 M.L.D. (0·1 c.c.)	Died overnight.
H	Nil	Nil	2 M.L.D. (0·1 c.c.)	Died overnight.

TABLE 12.—IMMUNISATION OF GUINEA-PIGS WITH "B. CHAUVŒI" FILTRATE.

Guinea-Pig.	Immunised.	Date.	Tested.	Date.	Result.
	"B. Chauvœi" Filtrate.		*1 M.L.D. "B. Chauvœi" Culture.*		
A	2·5 c.c.	17/7/23	0·5 c.c. I.M.	18/8/23	Lived.
B	2·5 c.c.	17/7/23	0·5 c.c. I.M.	18/8/23	Lived.
C	2·5 c.c.	17/7/23	0·5 c.c. I.M.	18/8/23	Lived.
D	2·5 c.c.	17/7/23	0·5 c.c. I.M.	18/8/23	Died 48 hours.
E	2·5 c.c.	17/7/23	0·5 c.c. I.M.	18/8/23	Lived.
F	2·5 c.c.	17/7/23	0·5 c.c. I.M.	18/8/23	Lived.
G	2·5 c.c.	17/7/23	0·5 c.c. I.M.	18/8/23	Lived.
H	2·5 c.c.	17/7/23	0·5 c.c. I.M.	18/8/23	Died 48 hours.
J	2·5 c.c.	17/7/23	0·5 c.c. I.M.	18/8/23	Lived.
Controls.			*1 M.L.D. C. Culture.*		
K	Nil	17/7/23	0·5 c.c. I.M.	18 8/23	Died overnight.
L	Nil	17/7/23	0·5 c.c. I.M.	18/8/23	Died overnight.
M	Nil	17/7/23	0·5 c.c. I.M.	18/8/23	Died overnight.
N	Nil	17/7/23	0·5 c.c. I.M.	18/8/23	Died overnight.

Table 13.—Immunisation of Guinea-Pigs with Two Doses of "B. Chauvœi" Filtrate, Subcutaneously.

Guinea-Pig.	1st Inoculation, 29/9/23.	2nd Inoculation, 2/10/23.	Tested.	Result.
	"B. Chauvœi" Filtrate.	*"B. Chauvœi" Filtrate.*	*"B. Chauvœi" Culture.*	
A	2 c.c.	2 c.c.	1 c.c. I.M. 15/10/23	Lived.
B	2 c.c.	2 c.c.	0·5 c.c. I.M. 29/10/23	Lived.
C	2 c.c.	1 c.c.	1 c.c. I.M. 15/10/23	Lived.
D	2 c.c.	1 c.c.	0·5 c.c. I.M. 29/10/23	Lived.
Controls.				
F	Nil	Nil	1 c.c. I.M. 15/10/23	Died 24 hours.
G	Nil	Nil	0·5 c.c. I.M. 29/10/23	Died 24–48 hours.
H	Nil	Nil	0·5 c.c. I.M. 29/10/23	Died 24–48 hours.
J	Nil	Nil	0·5 c.c. I.M. 29/10/23	Died 24–48 hours.

Immunisation of Guinea-Pigs against "Vibrion Septique" and "Bacillus Chauvœi" Simultaneously.

By inoculation of *V. septique* toxin antitoxin mixture combined with *B. chauvœi* filtrate we have succeeded in rendering guinea-pigs refractory to both organisms simultaneously.

EXPERIMENTS ON SHEEP.

No. 1. Immunisation against "Vibrion Septique."

Sheep A1. *Inoculated* on 28/11/23 with 5 c.c. of a 50 per cent. underneutralised *V. septique* toxin antitoxin mixture (T.A.T.) subcutaneously. *Tested* on 23/2/24 with 4 c.c. of *V. septique* liver broth culture injected intramuscularly (I.M.). *Result.*—Died a few hours after the Control Sheep E17.

Sheep A2. *Inoculated* on 3/12/23 with 5 c.c. of a 25 per cent. underneutralised *V. septique* T.A.T. mixture subcutaneously. The same dose was repeated on 19/2/24. *Tested* on 25/3/24 with 4 c.c. of *V. septique* liver broth culture I.M. *Result.*—The animal remained quite healthy and showed no lameness or other signs of reaction. Control Sheep E19 died within 24 hours.

Sheep A3.—*Inoculated* on 10/12/23 with 5 c.c. of a 25 per cent. underneutralised *V. septique* T.A.T. mixture subcutaneously. On 29/2/24 10 c.c. of *V. septique* toxin was injected subcutaneously. *Tested* on 25/3/24 with 4 c.c. of *V. septique* liver broth culture I.M. *Result.*—The animal proved to be immune. Control Sheep E19 died within 24 hours.

No. 2. Immunisation against "Bacillus Chauvœi."

Sheep D14. *Inoculated* on 28/11/23 with 5 c.c. of *B. chauvœi* filtrate subcutaneously. *Tested* on 25/3/24 with 3 c.c. of *B. chauvœi* liver broth culture I.M. *Result.*—Remained perfectly healthy. Control Sheep E20 died 24 to 36 hours after inoculation with the same dose.

Sheep D15. *Inoculated* on 28/11/23 in the same manner as Sheep D14, but not tested until 5/7/24, when it received 3 c.c. of *B. chauvœi* culture I.M. and survived without showing any untoward symptoms. For control sheep see E22.

No. 3. Simultaneous Immunisation against "Vibrion Septique" and "Bacillus Chauvœi."

Sheep B5. *Inoculated* on 17/12/23 with 5 c.c. of a 25 per cent. under-neutralised *V. septique* T.A.T. mixture and 5 c.c. of *B. chauvœi* filtrate subcutaneously. *Tested* on 23/2/24 with 4 c.c. of *V. septique* culture I.M. *Result.*—Died a few hours after Control Sheep E17.

Sheep B6. *Inoculated* on 17/12/23 with the same mixture as Sheep B5. The dose was repeated 29/2/24. *Tested* (*a*) on 25/3/24 with 3 c.c. of *B. chauvœi* culture I.M. *Result.*—Remained perfectly healthy. For control sheep see E20. On 5/4/24 the animal was tested for immunity against *V. septique* with 4 c.c. of *V. septique* culture I.M. *Result.*—Survived without showing any symptoms. Control Sheep E21 died within 24 hours.

Sheep B7.—This animal was immunised and tested in the same manner and on the same dates as Sheep B6 with entirely similar results.

Sheep B8. *Inoculated* on 17/12/23 with 5 c.c. of a 25 per cent. under-neutralised *V. septique* T.A.T. mixture and 5 c.c. of *B. chauvœi* filtrate subcutaneously. On 29/2/24 a second immunising dose of 5 c.c. *V. septique* toxin and 5 c.c. *B. chauvœi* filtrate was given. *Tested* on 25/3/24 with 4 c.c. *V. septique* culture I.M. *Result.*—Remained perfectly healthy. For control sheep see E19. By an oversight it has not been tested for immunity against *B. chauvœi* at the time of this publication.

Sheep C9. *Inoculated* on 17/12/23 with 5 c.c. of a 25 per cent. under-neutralised *V. septique* T.A.T. mixture and 5 c.c. of *B. chauvœi* filtrate subcutaneously. The dose was repeated on 24/12/23. *Tested* (*a*) on 23/2/24 against *V. septique* with 4 c.c. culture I.M. *Result.*—Showed lameness which soon passed off, but no other untoward symptoms. For control sheep see E17. *Tested* (*b*) on 5/7/24 against *B. chauvœi* with 3 c.c. culture I.M. *Result.*—Remained perfectly healthy. For control sheep see E22.

Sheep C11.—This animal was immunised in exactly the same manner and on the same dates as Sheep C9. It was *tested* (*a*) against *V. septique* on 25/3/24 with 4 c.c. culture I.M. *Result.*—Showed nothing but a very slight lameness, which soon passed off. For control sheep see E19. *Tested* (*b*) against *B. chauvœi* on 5/7/24 with 3 c.c. culture I.M. *Result.*—Survived. Control Sheep E22 was killed at the point of death 24 hours after infection.

Sheep C12.—This animal was immunised in exactly the same manner and on the same dates as Sheep C9. *Tested* (*a*) on 25/3/24 against *B. chauvœi* with 3 c.c. culture I.M. *Result.*—Remained quite healthy. For control sheep see E20. *Tested* (*b*) on 5/4/24 against

V. septique with 4 c.c. culture I.M. *Result.*—It again remained quite healthy. For control sheep see E21.

CONTROL SHEEP E17. *Inoculated.*—23/2/24 with 4 c.c. *V. septique* culture (I.M.). Died within 24 hours.

CONTROL SHEEP E19. *Inoculated.*—25/3/24 with 4 c.c. *V. septique* culture (I.M.). Died within 24 hours.

CONTROL SHEEP E20. *Inoculated.*—25/3/24 with 3 c.c. *B. chauvœi* culture (I.M.). Died 24 to 36 hours later.

CONTROL SHEEP E21. *Inoculated.*—5/4/24 with 4 c.c. *V. septique* culture (I.M.). Died within 24 hours.

CONTROL SHEEP E22. *Inoculated.*—5/7/24 with 3 c.c. *B. chauvœi* culture (I.M.). Was killed at the point of death 24 hours after injection.

Numerous guinea-pig experiments were done simultaneously with those on sheep confirming the virulence of the cultures. We found that about ten times the guinea-pig minimum lethal dose of *B. chauvœi* culture was sufficient to kill sheep within 24–36 hours. Of the *V. septique* culture about eighty times the minimum lethal dose was necessary.

CONCLUSIONS.

From the results which we have thus obtained in sheep, the following conclusions may be drawn :—

1. That a single dose of *V. septique* T.A.T. mixture does not produce sufficient immunity to enable the animal to withstand inoculation with a certain fatal dose of culture after an interval of two months. See Sheep A1 and B5.

2. That two doses of such a mixture are efficient for this purpose up to five months at least.

3. That although a reasonably strong immunity is produced when the interval between the two immunising doses is as short as one week, yet it appears that it is an advantage to give a longer interval between these doses. As a practical measure we would suggest that a fortnight be allowed.

4. That a single dose of *B. chauvœi* filtrate affords good protection against a certain fatal dose of culture for a period of at least six months.

5. That two doses of a combination of these products immunises an animal simultaneously against both organisms.

We wish to thank those members of the veterinary profession who, in response to our appeal, have sent us material from cases of blackleg, and we gratefully acknowledge the help which we have received from our colleagues of the Wellcome Laboratories, and particularly from Dr. A. F. Watson, who has kindly superintended the preparation of the media which we have used.

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